EXHIBIT "D"

13

Avidin–Biotin Systems

One of the most popular methods of noncovalent conjugation is to make use of the natural strong binding of avidin for the small molecule biotin. The strength of the avidin–biotin interaction has made it a useful tool in specific targeting applications and assay design. Since each avidin molecule contains a maximum of four biotin binding sites, the interaction can be used to enhance the signal strength in immunoassay systems.

Modification reagents that can add a functional biotin group to proteins, nucleic acids, and other molecules now come in many shapes and reactivities (Chapter 8, Section 3). Depending on the functional group present on the biotinylation compound, specific reactive groups on antibodies or other proteins may be modified to create an avidin (or streptavidin) binding site. Amines, carboxylates, sulfhydryls, and carbohydrate groups can be specifically targeted for biotinylation through the appropriate choice of biotin derivative. In addition, photoreactive biotinylation reagents (Chapter 8, Section 3.4) are used to add nonselectively a biotin group to molecules containing no convenient functional groups for modification. In this manner, oligonucleotide probes often are modified for detection with avidin or streptavidin conjugates (Chapter 19, Section 2.3).

The following sections discuss the concept and use of the avidin–biotin interaction in bioconjugate techniques. Preparation of biotinylated molecules and avidin conjugates also are reviewed with suggested protocols. For a discussion of the major biotinylation reagents, see Chapter 8, Section 3.

1. The Avidin–Biotin Interaction

Avidin is a glycoprotein found in egg whites that contains four identical subunits of 16,400 daltons each, giving an intact molecular weight of approximately 66,000 daltons (Green, 1975). Each subunit contains one binding site for biotin, or vitamin H, and one oligosaccharide modification (Asn-linked). The tetrameric protein is highly basic, having a pI of about 10. The biotin interaction with avidin is among the strongest non-covalent affinities known, exhibiting a dissociation constant of about 1.3×10^{-15} M. Tryptophan and lysine residues in each subunit are known to be involved in forming the binding pocket (Gitlin *et al.*, 1987, 1988).

The tetrameric native structure of avidin is resistant to denaturation under extreme chaotropic conditions. Even in 8 *M* urea or 3 *M* guanidine hydrochloride the protein maintains structural integrity and activity (Green, 1963). When biotin is bound to avidin, the interaction promotes even greater stability to the complex. An avidin–biotin complex is resistant to breakdown in the presence of up to 8 *M* guanidine at pH 5.2. A minimum of 6–8 *M* guanidine at pH 1.5 is required for inducing complete dissociation of the avidin–biotin bond (Cuatrecasas and Wilchek, 1968; Bodanszky and Bodanszky, 1970). Since the subunits in avidin are not held together by disulfide bonds, conditions that cause denaturation also result in subunit disassociation.

The strength of the noncovalent avidin–biotin interaction along with its resistance to breakdown makes it extraordinarily useful in bioconjugate chemistry. Biotinylated molecules and avidin conjugates can "find" each other under the most extreme conditions to bind and complex. The biospecificity of the interaction is similar to antibody–antigen or receptor–ligand recognition, but on a much higher level with respect to affinity constants. Variations in buffer salts and pH, the presence of denaturants or detergents, and extremes of temperature will not prevent the interaction from occurring (Ross *et al.*, 1986).

The only disadvantage to the use of avidin is its tendency to bind nonspecifically to components other than biotin due to its high pI and carbohydrate content. The strong positive charge on the protein causes ionic interactions with more negatively charged molecules, especially cell surfaces. In addition, carbohydrate binding proteins on cells can interact with the polysaccharide portions on the avidin molecule to bind them in regions devoid of targeted biotinylated molecules. These nonspecific interactions can lead to elevated background signals in some assays, preventing the full potential of the avidin–biotin amplification process to be realized.

Streptavidin is another biotin binding protein isolated from *Streptomyces avidinii* that can overcome some of the nonspecificities of avidin (Chaiet and Wolf, 1964). Similar to avidin, streptavidin contains four subunits, each with a single biotin binding site. After some postsecretory modifications, the intact tetrameric protein has a molecular mass of about 60,000 D, slightly less than that of avidin (Bayer *et al.*, 1986, 1989).

The primary structure of steptavidin is considerably different than that of avidin, despite the fact that they both bind biotin with similar avidity. This variation in the amino acid sequence results in a much lower isoelectric point for streptavidin (pI 5–6) than the highly basic pI of 10 for avidin. Moderation in the overall charge of the protein substantially reduces the amount of nonspecific binding due to ionic interaction with other molecules. Of additional significance is the fact that streptavidin is not a glycoprotein; thus there is no potential for binding to carbohydrate receptors. These factors lead to better signal-to-noise ratios in assays using streptavidin–biotin interactions than those employing avidin–biotin.

Both avidin and streptavidin can be conjugated to other proteins or labeled with various detection reagents without loss of biotin binding activity. Streptavidin is slightly less soluble in water than avidin, but both are extremely robust proteins that can tolerate a wide range of buffer conditions, pH values, and chemical modification processes. Bioconjugate techniques can utilize the ε- or N-terminal amines on these proteins for direct conjugation or employ modification reagents, such as thiolation compounds, to transform their existing functional groups into other reactive groups (Chapter 1, Section 4).